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(54) A hepatitis B virus (subtype ayw) surface antigen variant

(57) We isolated and characterized a new surface mutant of the hepatitis B virus surface antigen (HBsAg). The mutant was isolated from a symptomatic patient

with Down's syndrome who was found to be persistently positive for both for HBsAg and anti-HBs Antibody (Ab) with an equally long-lasting anti-HB core (c) IgM Ab

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Description

[0001] Hepatitis B virus (HBV), a small double stranded DNA virus, can cause a wide spectrum of clinical presentations: asymptomatic carrier state, acute self-limited hepatitis, fulminant hepatitis, and chronic liver diseases including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. It has a circular genome of 3182 to 3221 base pairs (bp). Four major subtypes have been identified and can be differentiated by antibodies that recognize the different epitopes on the HBV surface. The HBsAg particles carry the common determinant, "a", as well as d or y and w or r subtype determinants, and are classified into the four major subtypes, i.e., adw, adr, ayw and ayr. Rare sera contain HBsAg particles with all four-subtype determinants (adywr). The antigenic determinants for the main HBV subtypes: adw, adr, ayw and ayr lie in the surface or "S" polypeptide. Two amino acid residues in particular, encoded by the S gene at codon positions 122 and 160, have been postulated to determine the different antigenic subtypes. While, the preC regions have frequently been reported to have mutations rendering HBe Ag negative. The virus has a high rate of mutation relative to other DNA viruses due to its mode of replication by reverse transcriptase of its pregenomic RNA. The importance of a novel mutant can be reflected in vaccine escape and HBsAg detection failure, implicating a public health problem.

Summary of the Invention

[0002] We have identified and characterized a new surface mutant of HBV. The mutant was isolated from a symptomatic patient with Down's syndrome who was found to be persistently positive for both for HBsAg and anti-HBs Antibody (Ab) with an equally long-lasting anti-HB core (c) IgM Ab. With a panel of six monoclonal antibodies (mAb (s)) to HBsAg, we evaluated the mutation influence on the major epitope of the "a" determinant antigenicity.

Brief Description of the Drawings

[0003] Figure 1 illustrates the phylogeny tree of the HBsAg genomes and the accession numbers. Sequence of S1 - S13 strains are obtained from reference (Norder H, Hammas B, Lofdahl S, Courouze AM, Magnus LO. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. J Gen Virol. 1992 May;73 (Pt 5):1201-8); GenBank accession numbers are given for the rest of strains.

Detailed Description

[0004] A sample was taken from a 43-year-old white male patient with Down's syndrome who presented with jaundice to the American University of Beirut Medical Center in March 1999. The patient had no history of immunization to HBV, no transmissible risk factor including blood transfusion, IV drugs, homosexuality, family HBV infection or hemodialysis. During investigation, he was found to have elevated liver function tests (LFT): ALT 450 IU/L (range 10-35 IU/L), AST 250 IU/L (range 10-40 IU/L), gamma-GT 383 IU/L (range 10-50 IU/L), Bilirubin was 3.8/2.6 mg/dL (total/direct) (range, total: 0.1-1.2 IU/L and direct: 0.0-0.2 IU/L). More significantly, we noted incongruity in the HBV-blood tests. The HBV markers test results using enzyme immunoassay (EIA) methodology were as follows: HBs Ag positive (>2/0.051), anti-HBs Ab positive (0.417/0.206), anti-HBc IgM Ab low positive (0.371/0.208), anti-HBc IgG Ab strong positive (>2/0.412), HBe Ag negative and anti-HBe Ab positive (0.197/0.830, a competitive assay). Both the anti-hepatitis A Ab and the anti-hepatitis C Ab were negative. Due to the discrepancy in the HBV markers, molecular methods were used to confirm the HBV antigen. The patient was continuously followed up for LFT that decreased consistently over the following ten months including AST 80 IU/L. However, the HBV markers that were repeated ten months after initial presentation revealed both HBsAg positive (>2/0.056) (DiaSorin, Italy) and anti HBs Ab positive (56/5) (BioMerieux, Mini-Vidas, France), and the persistence of a low positive anti-HBc IgM Ab (0.303/0.221) (DiaSorin, Italy).

Example 1: Extraction and Amplification of HBs DNA

[0005] DNA was isolated and purified from 40 μ L samples based on a guanidinium-thiocyanate (GuSCN) lysis method. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction was used with some modifications. Five volumes of lysis solution [5.75 M GuSCN, 50 mM Tris-Cl pH7.5, 100 mM 2-mercaptoethanol and 15 μ g/mL poly (A)] were added to 1 volume of serum. After a brief vortex, the mixture was heated at 60°C for 10 minutes. The DNA pellet was obtained with isopropanol precipitation, and was washed with 75% ethanol. Dried DNA pellet was then resuspended in 50 μ L of PCR reaction solution, with 40 μ M (each) of the first-round primers (F1-6 and R1-6). The mixture was heated to 95°C for 3 min, followed by 30 PCR cycles consisting of 94°C/45 sec, 55°C/45 sec and 72°C/45 sec in a thermal cycler (GeneAmp PCR System 9600, Perkin Elmer, USA). 0.5 μ L of the first-round PCR

product was then served as template for a second-round PCR amplification using F1-7 and R13-2d primers pair consisting same cycles except annealing temperature was raised to 60°C. Positive PCR products, a DNA band of 604bp as expected, were detected in agarose gel electrophoresis.

5 [0006] The PCR Primer sequences [nucleotide sequences were derived from HBV DNA (accession number J02203) were as follows:

First-round Primer Set:

10 F1-6 (231-252) : CCTCACAATACCGCAGAGTCTA

R1-6 (816-842) : AGGCTTTAAATGTATAACCCAAACACAA

15 Second-round Primer Set:

F1-7 (246-268) : GAGTCTAGACTCGTGGTGGACTT

20 R13-2d (822-843) : ccgaattcTAGGGTTAAATGTATAACCCAA

[0007] An EcoR I site was added at the 5'-end of R13-2d for cloning purpose.

25 Example 2: Sequence Alignment and Phylogenetic Analysis

[0008] Amplified PCR product was cleaned with a QIAquick spin column (Qiagen) and subsequently cloned for DNA sequencing and protein expression. Five clones were sent for DNA sequencing in order to obtain reliable DNA sequence determination. Nucleotide sequences were determined for both strands with the BigDye Terminator Ready Reaction 30 Kit (PE Applied Biosystems, USA) on an ABI 377 DNA Sequencer (PE Applied Biosystems, USA). Sequence analysis was performed using SeqMan 4.00 module of the Lasergene package (DNAStar Inc., Madison, WI, USA). Sequence alignment and the construction of phylogenetic trees were computed by MegAlign 4.00 module of the Lasergene package (DNAStar Inc., Madison, WI, USA). Clustal multiple sequence alignment was used through sequence weighting. The 28 reference strains for genotype grouping were derived from published sequences.

35 Example 3: S-HBsAg Cloning and Transient Protein Expression

[0009] The PCR product was digested by Xba I /EcoR I restriction enzymes. The 595 bp fragment encoded 86% of the S-HBsAg protein from amino acids Leu 32 to the end (Ileu 226). The fragment was then ligated into a previously 40 constructed mammalian expression vector, to replace the wild-type ayw S-HBsAg fragment, which was placed downstream of a CMV promoter. The transfection was then performed on a COS-7 cell line using LIPOFECTAMINE Plus reagent (Life technologies, MD, USA). Culture supernatant containing secreted variant S-HBsAg from the COS-7 cell infections was then harvested and fresh medium was added every 72 hours after transfection. Wild-type ayw [wt/ayw] S-HBsAg, (GeneBank accession number J02203), was also expressed for control use.

45 Example 4: Recombinant HBsAg Antigen Immunoassay and Epitope Analysis

[0010] Recombinant wt/ayw and LBN variant S-HBsAg expression was determined by solid-phase EIA in a sandwich format. One polyclonal and a panel of six monoclonal antibodies [mAb(s)] were used in this study. They were goat anti-HBsAg (ay/ad) (Fitzgerald, MA, USA), HB-1, HB-8, HB-9, HB-13, HB-14 and HB-16. All six mAb(s) were raised against serum derived wild-type HBsAg and were reactive to both ad and ay subtypes. EIA were used to measure serum and recombinant HBsAg reactivity. Basically, purified mAb(s) or polyclonal anti-HBsAg were coated on a microtiter plate and kept at 4°C until use. Prior to performing EIA, the plates were blocked with PBS containing BSA for 2 hours at 50 25°C. EIA was performed in a one-step sandwich format with 150 μ L of sample (serum or diluted recombinant HBsAg culture) mixed with 50 μ L HRP conjugated monoclonal antibodies. The plates were incubated at 37°C for 90 minutes, then washed 6 times with PBS containing 0.005% Tween 20. The plates were developed by a final incubation for 30 minutes with 200 μ L of OPD solution (Sigma, USA). Adding 50 μ L of 4N Sulfuric acid then stopped the color development. The absorbance was read on an automatic microtiter plate reader (Molecular Devices, USA) and the results were

expressed as optical density (OD) units at 490 nm. To evaluate the mAb reactivity to the native HBsAg and to the synthetic peptides, direct EIA procedure was performed. A serial dilution of each purified and quantity adjusted mAb was incubated on plates, which were previously coated with purified native HBsAg or oxidized peptides. Bound murine IgG were detected by a second incubation with horseradish peroxidase conjugated rabbit anti-mouse IgG. The reactivity was ascertained by enzyme catalyzed OPD color development. Recombinant variant and wild-type S-HBsAg were also analyzed by western immunoblot. HBsAg culture supernatant was concentrated 15 fold by a centrifugal filter (Biomax-100, Millipore, USA). The concentrate was run on a NuPAGE 10% Bis-Tris SDS Gel (Novex, San Diego, USA) and then transferred to nitrocellulose membrane. Transferred protein was immunoblotted with HBsAg mAb. The binding was detected using an immunoblot system (BioRad, GAM-AP, USA).

Example 5: HBs Variant DNA Sequence and Amino Acid Sequence Analysis

[0011] The HBs DNA sequence of this novel Lebanese (LBN) variant was analyzed for genotypes. Sequence comparison between variant HBs DNA sequence and other 28 defined HBs genotype strain put this variant HBs into genotype D group (Fig. 1). Although serological typing on this patient's serum was not performed, the amino acid sequence of this HBs variant showed highest homology with ayw2 HBs subtype. Five amino acid mutations within position 32-226 of LBN variant were observed in all five clones. They are 103 M (ATG)→I (ATT), 118 T (ACG)→K (AAG), 120 P (CCA)→Q (CAA), 170 L (TTA)→S (TCA) and 213 L (TTA)→S (TCA). At amino acid position 122, three clones revealed sequence Arg (AGA) while 2 clones revealed sequence as Lys (AAA). For recombinant LBN HBsAg expression, sequence with Arg (122) was used.

102	-	110	-	120	-	130	-	140	-	150
25	wt (ayw2)	GMLPVCPLIPGSSTTSTGPCRTCTTPAQGTSNYPSCCCTKPSDGnCTCI								
	LBN	-I-----K-Q-----								

[0012] Wild-type HBsAg consensus sequence was derived from other three strains (X02496, M32138 and S7) of the genotype D group. Bold indicates the "a" determinant region (Stirk HJ, Thornton JM, Howard CR. A topological model for hepatitis B surface antigen. *Intervirology*. 1992;33(3) :148-58)

Example 6: Recombinant HBsAg Transient Expression in COS-7 Cell

[0013] The secreted recombinant HBsAg expression was in a range of 0.1-0.2 mg/liter, calibrated by a purified wild-type recombinant HBsAg. Only secreted HBsAg in culture supernatant was examined for expression. There was no significant expression yield difference at 72 hours and 144 hours time point. The culture supernatant harvested at 216 hours gave only 1/5 expressed HBsAg compared to the supernatants collected at 72 and 144 hours.

Example 7: Immunoreactivity Analysis:

[0014] Both the recombinant HBsAg (ayw) and the novel variant HBsAg (LBN) culture supernatants were tested for their reactivity by a panel consisting of six mAb(s). Culture supernatants were diluted 1:40 to insure the same quantity of antigen concentration. Amino acid mutations apparently affected four mAb(s) binding affinity to wt HBsAg. HB-16 was affected most, losing almost 90% strength. Strikingly, the binding strength of two mAb(s), HB-13 and HB-14, to the LBN variant was significantly increased to 5-6 fold. Peptide mapping revealed that these two antibodies were specific to the first loop linear sequence while mAb HB-9 was specific to the sequence from 137 to 147, located in the second loop region. All the three mAb(s), HB-1, HB-8 and HB-16, are conformation specific. They did not react with SDS denatured HBsAg in immunoblot but had higher dilution titer in direct EIA assay. On the other hand, the mAb(s) HB-13 and HB-14 blotted to both wild type and LBN HBsAg very well, indicating that these two mAb(s) recognize denatured epitopes, independent of the mutation sites.

Table 1.
Immuno reactivity of a panel of monoclonal antibodies to various antigens in E/A assay and in western Immunoblot

		Monoclonal antibodies raised against wild type HBsAg					
		HB-1	HB-8	HB-9	HB-13	HB-14	HB-16
mAb relative reactivity (%) to LBN variant *	55.5	55.7	35.3	530.1	665.2	10.3	
mAb titer against native HBsAg **	1:8X10 ⁵	1:2X10 ⁵	1:2X10 ⁴	1:4X10 ⁴	1:4X10 ⁴	1:4X10 ⁵	
peptide (122-137) #	0.161	1:50	0.164	1:50	0.125	1:50	>2.00
peptide (139-147) #	0.183	1:50	0.223	1:50	>2.00	1:500	1:50
peptide (122-147) #	0.201	1:50	0.261	1:50	>2.00	1:2K	0.292
Western Immunoblot	WT	(AYW) LBN	-	-	n.p.	++	+++
			-	-	n.p.	++	+++

* Relative reactivity was presented as OD derived from each mAb reactivity to LBN variant compared with OD derived from its reactivity to (AYW) which was set at 100%.

** mAb titer was expressed as dilution needed for each mAb to obtain a preset signal between OD=1.00 to 1.25 in a direct E/A with native HBsAg.

Reactivity was expressed as OD obtained from each mAb at indicated dilution. All mAb(s) concentration was adjusted at a same concentration start point. All peptide sequence was based on wt(AYW).
n.p. : not performed. Western Immunoblot results were visually determined.

Claims

1. An isolated variant hepatitis B surface antigen comprising an amino acid sequence wherein mutations from hepatitis B wild type ayw2 strain appear as follows: at position 103 isoleucine is present instead of methionine, at position 118 lysine is present instead of threonine, at position 120 glutamine is present instead of proline, at position 170 serine is present instead of leucine, and at position 213 serine is present instead of leucine.
2. An expression vector for expression of a variant hepatitis B surface antigen in a recombinant host, wherein said vector contains a recombinant gene encoding the variant hepatitis B surface antigen of claim 1.
3. A monoclonal antibody raised against the variant hepatitis B surface antigen of claim 1.
4. A hybridoma cell line which secretes the monoclonal antibody of claim 3.
5. An assay kit for determining the presence of hepatitis B in a test sample, comprising: a container containing at least one monoclonal antibody which specifically binds to hepatitis B surface antigen wherein the monoclonal antibody is a monoclonal antibody secreted by the hybridoma cell line claimed in claim 4.
6. A method for determining the presence of hepatitis B in a test sample, comprising:
 - a. contacting a test sample with at least one monoclonal antibody claimed in claim 3 attached to a solid phase, to form a mixture,
 - b. incubating the mixture for a time and under conditions sufficient to form antigen-antibody complexes,
 - c. contacting the complexes with an antibody conjugated to a signal generating reagent that is specific for the complexes, and
 - d. determining the presence of hepatitis B present in the test sample by detecting the signal generated.

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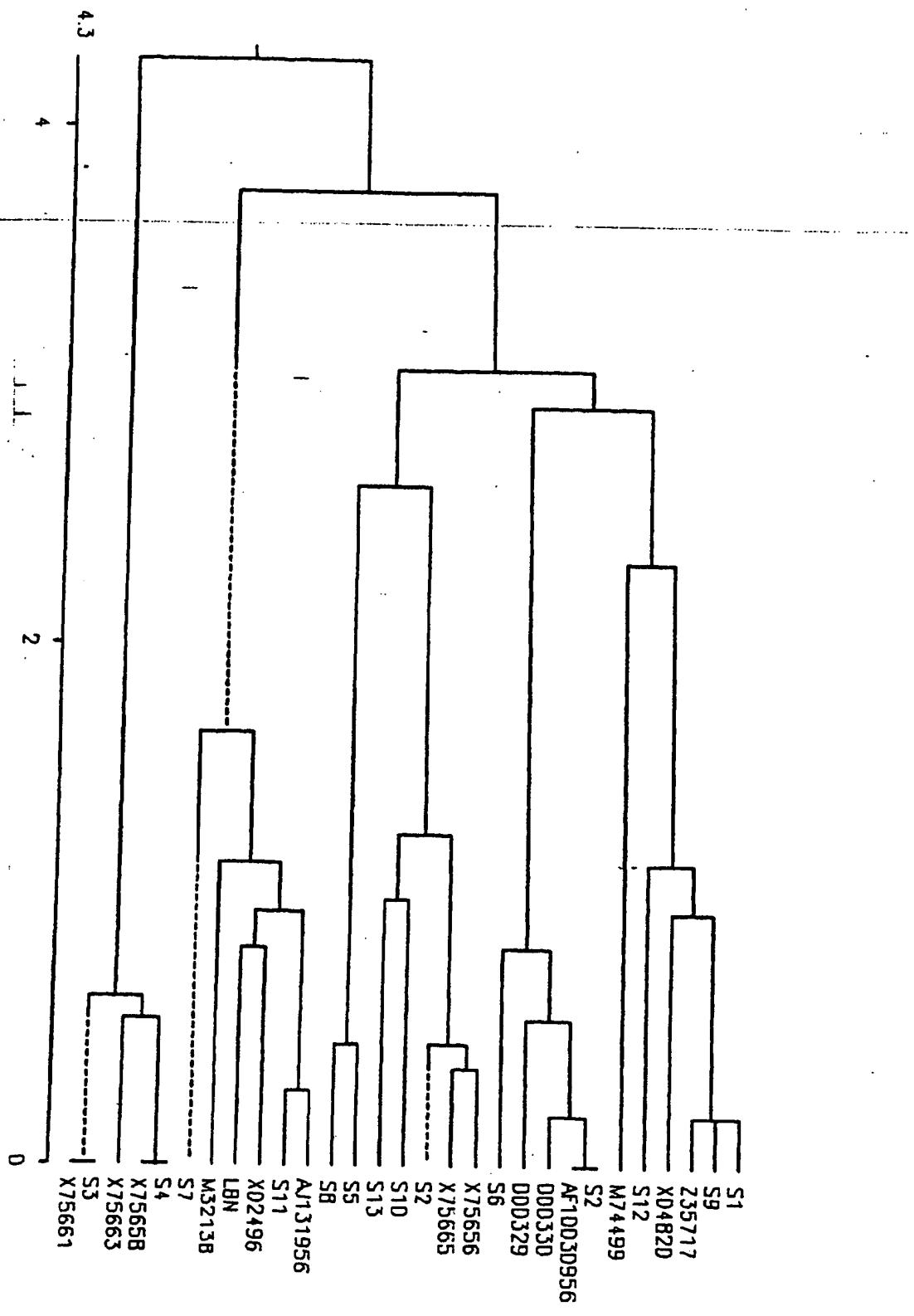


Fig. 1



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PARTIAL EUROPEAN SEARCH REPORT

Application Number

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proceedings, as the European search report

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (HCL7)												
X,D	<p>DATABASE EMBL 'Online! EMBL; Accession no. J02203, 9 June 1982 (1982-06-09) "Hepatitis B virus (strain ayw) genome" XP002173119 * abstract *</p>	1-6	<p>C07K14/08 A61K39/42 C07K16/10 G01N33/576</p>												
X	<p>DATABASE EMBL 'Online! EMBL; Accession No. AAF208877, 1 January 2000 (2000-01-01) WIENBERGER ET AL.: "Hepatitis B virus isolate wr2209 small surface antigen (s) gene, complete cds" XP002173120 * abstract *</p>	1-6 -/-													
INCOMPLETE SEARCH															
<p>The Search Division considers that the present application, or one or more of its claims, does not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.</p> <p>Claims searched completely :</p> <p>Claims searched incompletely :</p> <p>Claims not searched :</p> <p>Reason for the limitation of the search:</p> <p>see sheet C</p>															
<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 33%;">Place of search</td> <td style="width: 33%;">Date of completion of the search</td> <td style="width: 33%;">Examiner</td> </tr> <tr> <td>MUNICH</td> <td>3 August 2001</td> <td>Marinoni, J-C</td> </tr> <tr> <td colspan="3" style="text-align: center;">CATEGORY OF CITED DOCUMENTS</td> </tr> <tr> <td colspan="3"> <p>X : particularly relevant; if taken alone Y : particularly relevant; if combined with another document of the same category A : technological background D : non-essential disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>S : member of the same patent family, corresponding document</p> </td> </tr> </table>				Place of search	Date of completion of the search	Examiner	MUNICH	3 August 2001	Marinoni, J-C	CATEGORY OF CITED DOCUMENTS			<p>X : particularly relevant; if taken alone Y : particularly relevant; if combined with another document of the same category A : technological background D : non-essential disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>S : member of the same patent family, corresponding document</p>		
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European Patent
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SHEET C

Application Number

EP 01 30 3073

Claim(s) searched completely:

Claim(s) searched incompletely:
1-6

Reason for the limitation of the search:

Present claim 1 relate to an extremely large number of possible polypeptides (i.e. all hepatitis B surface antigens having the 5 mutations referred to in claim 1). In fact, the subject-matter of the claim is directed to so many possible polypeptides that a lack of clarity (and conciseness) within the meaning of Article 84 EPC arises to such an extent as to render a meaningful search of the claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear (and concise), namely the variant of the Hepatitis B surface antigen having the sequence of EMBL accession number J02203 and having all 5 mutations referred to in claim 1.

Additionally, it is noted that the sequence of J02203 has a Phe at position 170 and not a Leu. The search has however been carried out based on the features of the resulting polypeptide.



PARTIAL EUROPEAN SEARCH REPORT

Application Number

EP 01 30 3073

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.)
Category	Citation of document with indication, where appropriate, - of relevant passages	Relevant to claim	
X,D	<p>NORDER H. ET AL.: "Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains" J. GEN. VIROL., vol. 73, 1992, pages 1201-1208, XP002140252 * the whole document *</p>	1-6	
X	<p>NORDER H. ET AL.: "Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene" INTERVIROL., vol. 38, 1995, pages 24-34, XP000197124 * the whole document *</p>	1-6	<p>TECHNICAL FIELDS SEARCHED (Int.Cl.)</p>

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